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Responses of citrus rootstock ovules to colchicine applications *In vitro*

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Abstract An *in vitro* study was conducted to produce autotetraploid citrus rootstocks using various colchicine levels. Immature fruits from open pollinated flowers of the four rootstocks viz. Sacthon Citrumello, Carrizo Citrange, Yuma Citrange and Brazilian Sour Orange were harvested 12 to 14 weeks of post-anthesis. Undeveloped ovules of each rootstock were cultured on the Murashige and Tucker basal medium supplemented with 500 mg/l malt extract. Various colchicine levels including 0.00, 0.01 and 0.10% were tested. Ovules were transferred to colchicine-free medium three weeks post inoculation and every eight weeks subsequently. Germinated embryos with roots and shoots were acclimatized and planted in pots. Results showed that an inverse relationship existed between seed germination and colchicine application. Plantlet regeneration from all citrus rootstocks varieties was suppressed dominantly at 0.1% level of colchicine. Morphological and cytological parameters were studied for confirmation of polyploidy. Chromosome study revealed 8 tetraploid plants with 0.10% treatment and 3 tetraploid with .01% treatment were found.

Key words: polyploidy, citrus, rootstocks, colchicines, *In* vitro

Introduction

Citrus trees are generally diploid species chromosome number 2n=18, which easily cross among genera producing fertile hybrids. Nearly all commercial citrus trees in the world are grown as grafted trees. An excellent scion and rootstock combination supports development of tree that bears large quantities of high quality fruit. Such a successful combination can maintain vigor and productivity for 50 years or more with modest supervision. Rootstock has much influence on the scion budded or grafted. However, many offered rootstocks are inadequate to meet the emerging needs and challenges. A large proportion of the troubles faced by the citrus industry could be defeat by using

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better rootstocks (Bowmann, 2000). Citrus rootstocks are propagated by polyembryonic seeds. Polyembryony arises from adventitious embryogeny from nucellar cells of the ovule and results in partial apomixis. The majority of rootstocks are apomictic, which can produce uniform plants from seed at a low cost. Several serious obstacles exist that hinder citrus hybridization. For example i) citrus is exceedingly heterozygous ii) its inimitable reproductive biology iii) pollen and ovule sterility causing incompatibility iv) long juvenile stage and v) adventitious embryos in the nucellar tissue of developing ovules minimize hybridization. Most of the present day scion and rootstock cultivars of citrus are the progeny of chance seedlings or a mutant branch of a tree, called 'bud sports'. Pakistan is still restricted to use only two rootstocks i.e., rough lemon (*C. jambhiri* L.) and sour orange (*C. aurantium* L). Problems arising by using these two rootstocks are Phytothora root rot or gummosis and Citrus Tristeza Virus (CTV). In such circumstances we are in terrible need to exploit other rootstocks which can handle the problems and citriculture can also be practiced under poor soil conditions. One of the solutions is to build up polyploid citrus rootstocks. As polyploid rootstocks have opportunities to adapt harsh conditions due to enzyme multiplicity; allelic diversity and enhanced production of secondary metabolites.

Polyploidy is the condition of some biological cells and organisms manifested by the presence of more than two homologous sets of chromosomes. Research interest in polyploids has been renewed in the past decade following the discovery of multiple origins and patterns of polyploid creation. Polyploidy in crop plants is most commonly induced by treating seeds with the chemical colchicine. Two basic mechanisms are involved in spontaneous polyploidization in citrus are 1) duplication of chromosome stocks in nucellar tissues that give rise to autotetraploids 2) 2n gametes arising mostly from second division restitution during meiosis of the megaspore that produce triploid hybrids in diploid ×tetraploid hybridization. Several polyploid genotypes were detected early on in citrus germplasm. Often the polyploid plants are better due to increased complement of the chromosomes (Gao *et al*., 1996). Citrus trees are subject to several abiotic constraints such as salinity and water logging. These polyploid rootstocks can be used in citrus growing areas as well as in water scarce and saline soils as it has been studied that tetraploid citrus rootstocks are more tolerant to salt stress than diploid (Saleh *et al.,* 2008). So the present study was carried out to develop such tetraploid citrus rootstocks by using colchicine. Three Poncirus hybrids have been used in this study as genus Poncirus is considered to be naturally salt excluder and this criteria is heritable also. Ovules of 14-16 weeks are usually used as embryos were at unicellular stage, so there were maximum chances for the evolution of

noncytochemeric plants. The main objective of the present study was to discover the possibility of induction of polyploidy by colchicine application and to develop tetraploid for diversification of the available citrus germplasm.

Materials and methods

Immature fruits from various rootstocks viz. Sacthon Citrumello, Carrizo Citrange, Yuma Citrange and Brazilian Sour Orange were randomly taken from the prescribed rootstocks and dipped in 95% alcohol just for a minute and flamed to remove surface contamination. Undeveloped ovules (14-16 weeks) of each variety were excised from these immature fruits and surface-sterilized using 95% alcohol for 4-5 minutes followed by washings with sterilized water. The entire process of sterilization was carried out under laminar flow hood in inoculation room to avoid contamination. The sterilized ovules were cultured on MT media (Murashige and Tucker, 1969) supplemented with 500 mg L^{-1} malt extract and various applications of colchicine. The colchicine levels were 0.00, 0.01, and 0.10 % on the basis of weight by volume (w/v).The cultures were then placed in growth room at 25± 2ºCwith 16 hour photoperiod. Ovules with subsequent proliferation were transferred to fresh medium without colchicine three weeks post-inoculation and every 8 weeks subsequently. The pH of media was adjusted at 5.7. Media was autoclaved after capping the test tubes at 121ºC temperature, under 15 lbs psi for 20 minutes.

Fifteen cultures of each colchicine treatment along with three replications were arranged in Completely Randomized Design (CRD). The obtained data was statistically analyzed by using statistix 8.1 package. Mean were compared by using Ducan's Multiple Range Test (DMRT) at significance level of 0.05. The observations recorded for percentage of sprouting and mortality, shoot length (cm), leaf area (cm^2), stomatal density, length/width of stomata (µ), chloroplast and chromosomal count. Germination and mortality percentage was calculated on the basis of total number of cultured plants per colchicine treatment. Shoot length and leaf area was calculated by measuring methods.

Stomatal and chromosamal studies

Stomatal and chromosomal studies were performed using microscope with staining method (by using fixative solution). For stomatal study a thin epidermal layer of the lower leaf surface was removed and mounted on a slide having a drop of distilled water. A drop of safranin solution was also added to stain the stomata and covered with a cover slip. Gentle pressing was done for the penetration of dye in the epidermal tissue. The one drop of xylene was placed on a cover slip to clear vision. Stomata were counted under x 1250

magnification. However the length was measured by the ocular micrometer adjusting length wise and width was measured placing in cross sectional position. The divisions were counted and multiplied with the factor one micron (Khan *et al.,* 1992a). For chromosomal counting, shoot tips from actively growing region of about 5-10 mm length were excised from young leaves of plants. Then washed excised segments were immersed in fixative solution containing ethyl alcohol (95%), glacial acetic acid and chloroform (6:1:3, v/v) for 24 hr at 4ºC temperature. Staining was done by using acetocarmine staining solution.

Chloroplast count in guard cells

The chloroplast count /guard cell was recorded by using piece of lower epidermal leaf (peeled off) and putting on the glass slide having a drop of water. The slide was observed under microscope at 10×40X magnification. Number of chloroplasts /guard cell was counted.

Results and discussions

Morphological studies

The significant differences were observed for germination among various citrus rootstocks. Each rootstock responded differently at various colchicines concentrations. Although all rootstocks exhibited better germination percentage more than 80% where no colchicine was applied. However, only the rootstock "Brazillian Sour orange*"* showed the highest response (92.33%) in response to the least level of 0.01% colchicine (Fig 1). These results reduced with increasing levels of colchicines (0.1%) in the same genotype. This may be possible due to genotypic differences. These results show that an inverse relationship exists between various colchicine concentrations and seed germination. These results are accordance with the findings of Duren *et al*., (1996) who found the negative effect of high colchicine concentration on germination percentage of explants. These results are also in agreement with *in Vitro* studies of Chakarborti *et al.* (1998) who used other plants types.

Mortality percentage of the germinated plantlets increased with increasing levels of colchicines in each genotype of the citrus (Fig 2). More than 50% of germinated plantlets were observed mortal at the highest levels of colchicine (0.10%). However, the lowest mortality percentage was observed where no colchicine was applied.

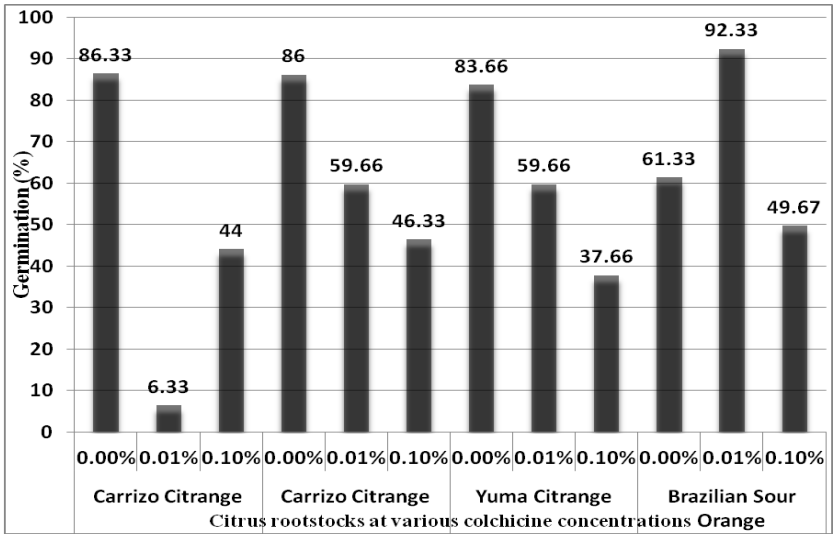

Fig. 1. Effect of various colchicines concentrations on the germination percentage of various citrus rootstocks.

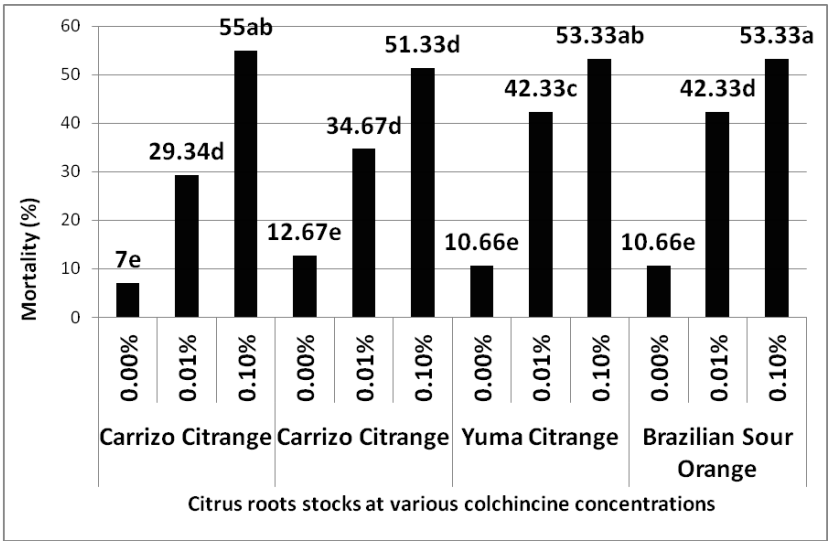

Fig. 2. Effect of various colchicine concentrations on the mortality (%) of various citrus rootstocks.

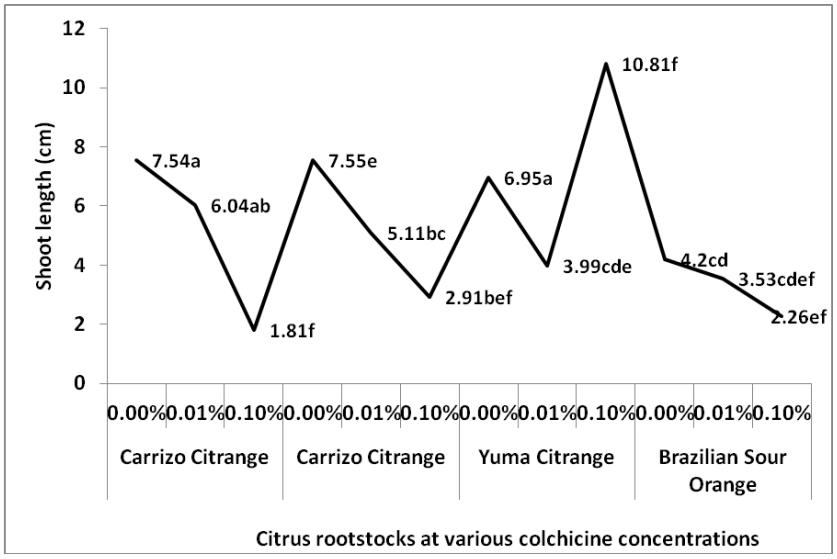

Fig. 3. Shoot length of various rootstocks as affected by colchicines treatments

Shoot length was significantly affected by various colchicines treatments. Maximum shoot length (10.81) was observed in Yuma Citrange rootstock in response to the highest colchicine (0.10%) treatment. However, rest of the rootstocks showed their best response where no colchicine treatment was applied and decreased shoot length with increasing levels of colchicine. The results indicate that colchicine had adverse and suppressing effects in most of the rootstocks. Similar observations in which shoot length decreased due to initial retardation of growth were also reported by Skidar and Jolly (1994). High concentration of colchicine coupled with long exposure time produced plants with desirable characters such as stunted growth (Rubuluza *et al*., 2007).

Leaf area was measured on the basis of diploid and tetraploid (Table 2). The leaf area increased with colchicine application. Leaf area was almost double in colchicine induced plants then their respective diploids. Maximum leaf area (19.84 cm^2) was observed in tetraploid of *Brazilian Sour Orange* (19.84cm^2) followed by 19.38 cm^2 in Sacthon Citrumello. However each rootstock showed the highest leaf area in tetraploid. Theiler-Hedritch (1991) in cherry also reported increased leaf area after colchicine treated plants than the untreated ones.

Cytological Studies

The study revealed that more number of stomata was observed from the leaves of diploid plants as compared to the triploid plants in each of citrus rootstock (Table 1). Maximum number of stomata (35.38) was observed from

Carrizo Citrange rootstock followed by 34.44 from *Yuma Citrange.* However width and length of stomata was found directly proportional to increase in ploidy level. The tetraploid plants were observed to be significantly higher for stomatal size as compared to diploid plants. The maximum stomatal length (31.35 µ) was observed from *Brazilian Sour Orange* rootstock followed by 29.31 µ in *Sacthon Citrumello*. However, the maximum stomatal width 23.01µ was observed in *Yuma Citrange.* The frequency, number and size of stomata have been useful for comparision of tetraploids, triploids and diploids. This study strengthens our finding which also shows direct relationship of stomatal size. However, stomatal number decreased with increase in ploidy level (Bashir, 1994; Khan *et al*., 1992; Jaskani, 2005, Usman *et al*., 2008).

Chloroplast number per unit area was significantly differed in stomata of leaves of treated plants as compared to the controlled plants. Maximum number of chloroplast (13.98) was observed in treated plants of Carrizo Citrange as compared to untreated plants (Table 2). The frequency of stomata reduced significantly with increasing ploidy level (Gu *et al*., 2005). The results are in conformity with the findings of Barret (1974), Takidze and Demetradze (1988) and khan *et al.*, (1992) who observed less number of stomata per unit area in tetraploid citrus leaf than that of diploid one.

Table 1. Stomatal studies of diploid and tetraploid citrus rootstocks as affected by various colchicines concentrations

	Sacthon Citrumello		**Carrizo Citrange**		**Yuma Citrange**		**Brazilian Sour Orange**	
	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid
Stomatal length (µ)	21.11±3.94	29.34±1.33	20.33±3.94	28.24±1.33	2.85±3.94	28.91±1.33	29.01±3.94	31.35±1.33
Stomatal width (µ)	17.06±1.39	20.67±0.99	19.67±1.39	22.29±0.99	18.52±1.39	23.01±0.99	16.62±1.39	21.68±0.99
Stomatal density (µ)	29.84±2.71	21.51±1.35	35.38±2.71	23.21±1.35	34.44±2.71	23.03±1.35	31.48±2.7	20.34±1.35

Table 2. Leaf area and chloroplast counts of diploid and tetraploid citrus rootstocks as affected by various colchicines concentrations.

	Sacthon Citrumello		**Carrizo Citrange**		**Yuma Citrange**		**Brazilian Sour Orange**	
	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid
Leaf Area (cm^2)	13.74±4.86	19.38±5.67	1.39±4.86	7.92±5.67	6.49±4.86	12.99±5.67	13.74±4.86	19.84±5.67
Chloroplast counts	9.67±1.45	13.1±0.69	9.8±1.45	13.98±0.69	6.69±1.45	12.52±0.69	8.96±1.45	12.5±0.69

Chromosome counts in mitotic cells of root tips and shoot tip is an accurate procedure to dtermuine the ploidy, but it is time consuming and requires much experience (Silva *et al*., 2000). The determination of

chromosome number in the roots of *in vitro* produced polyploid plants is very convenient and effective in comparison with those of field plants (Gao *et al*., 1996). Diploid citrus plants possessed 18 chromosomes. In control (0%) treatment 18 chromosomes were found which found confirmed their diploid status. During this study 8 tetraploid plants with (2n=36) at 0.10% treatment and 3 tetraploid at 0.01 % treatments were found.

Conclusion

Polyploid induction in citrus rootstocks is desired to promote citrus cultivation in saline and water logged soils as well as to overcome the obstacles which our citrus industry is facing due to rootstocks. This was a first attempt regarding this. Carrizo Citrange, Yuma Citrange, Sacthon Citrumello were used during this study as they are naturally salt excluders. These three are hybrids of Pocirus in which this character is heritably transmitted. Immature fruits were used in this study in which Embryo was at unicellular stage, so there were more chances of noncytochimeric plants development.

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